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**Hypocrellin B, a perylenequinonoid pigment, and its complexes with lanthanide ions:
optical characterization and enhancements in its photodynamic properties**

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Hypocrellins, perylenequinonoid pigments isolated from *Hypocrella bambusae*, are known photodynamic agents. They exhibit several advantages over the other photosensitizers, such as ready preparation, easy purification, low aggregation tendency, high singlet oxygen generation quantum yield and rapid metabolism *in vivo*. However, hypocrellins do not exhibit absorption at wavelengths longer than 600 nm, which limits their application for PDT. To solve this problem our efforts were focused towards modifying the properties of hypocrellin B. In this study, hypocrellin B complexes formed with the lanthanide ions lanthanum (La^{3+}), terbium (Tb^{3+}) and europium (Eu^{3+}), using ethanol as solvent, had their optical properties characterized through spectroscopic methods. We observed that all the lanthanide ions change the HB molecular structure, since displacements in the bands were noted in both absorption and emission spectra. Different molar ratios between the HB and the lanthanide ions were tested, and it was verified that the complexes with 1 mol of HB for 2 mol of lanthanide ions present the larger red-

shift comparatively to pure HB. We also determined the dimerization constants for these complexes. Under excitation at 532 nm the singlet oxygen generation quantum yields were estimated by the emission at 1270 nm, where singlet oxygen has a well known emission peak. It can be affirmed that such complexations were responsible for an improvement in the optical properties of the HB molecule, which suggests that these complexes may find a good application at *in vivo* studies.

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1. Introduction

The treatment of cancer is one of the major challenges in medicine, and the possibility of new methods for diagnosis and therapy is being persecuted worldwide. Photodynamic therapy (PDT) is a promising modality^{1,2} to cancer treatment.

PDT action initiates when an organic dye (photosensitizer) absorbs light in the range 600-900 nm, the so-called therapeutic window, in which the light scattering by tissues is low and there is a small competing absorption from water and body chromophores such as hemoglobin, melanin, etc. After the light is absorbed, the dye must generate Reactive Oxygen Species (ROS), upon photochemical reaction with non absorbers molecules presents in the environment. These ROS are responsible for the apoptosis in the target tissues³. The photochemical reactions occur under electron transfer (type I), generating radical products, and/or energy transfer (type II), generating singlet oxygen⁴.

PDT depends also of conditions as light exposure interval, light exposure regime, photosensitizer concentrations, environment, etc⁵. Between several dyes used nowadays for PDT there is the Hypocrellin B (HB), shown in Figure 1, a naturally perylenequinonoid dye extracted from the fungus of *Hypocrella Bambusae*^{3, 6-8}. The HB dye in ethanol absorbs light at 590 nm, and its singlet oxygen generation quantum yield is 0.76⁹.

Ma et al.⁹ demonstrated the possibility of improving the performance of HB complexing aluminum in its structure. In this case, the absorption band is shifted to around 618 nm in DMSO, but the singlet oxygen generation quantum yield is lowered to 0.23. The results indicate that this complex possesses an enhanced type I process but a decreased type II process when compared with HB.

In this work we present studies of HB complexation with the lanthanide ions europium, terbium and lanthanum. This investigation is inspired in a published work where lanthanum ions were shown to improve the PDT properties of hypocrellin A dye¹⁰. The main objective of this work is to obtain a photosensitizer that is easily prepared, with good solubility, purity, good absorption band in the therapeutic window, presenting a high quantum yield of triplet formation and singlet oxygen, and minimal side effects (photosensitivity and toxicity).

2. Materials and Methods

2.1. HB:Ln³⁺ complexes and best molar ratios

Hypocrellin B (HB) was purchased from Shaanxi Tianze Bio-Technology Co., Ltd.; analytical grade Methylene blue (MB), lanthanide chlorides (LaCl₃·7H₂O, EuCl₃·6H₂O and TbCl₃·6H₂O) were purchased from Sigma-Aldrich Corporation Ltd. HB and the lanthanide

chlorides (Ln^{3+}) were diluted in ethanol, forming solutions of equal molarity. HB and Ln^{3+} solutions were mixed following the molar ratios 1:0.5, 1:1, 1:2 and 1:3, aiming to determine the number of moles of Ln^{3+} that chelates on the HB molecule. It is important to note that all prepared solutions had the same concentration, 70 μM of HB, allowing the quantitative comparison of the complexes optical properties.

2.2 Optical characterization of the complexes - absorption and emission spectra

The absorption spectra of all samples were measured at room temperature in the range 300 nm-700 nm using a Varian Cary 17D Spectrometer. The spectra were plotted in terms of molar absorptivity (ϵ):

$$\epsilon = \frac{OD}{b \cdot c} \quad (1)$$

where OD is the optical density, b represents the optical path (0.1 cm) and c is the solution concentration (70×10^{-6} M), and ϵ is given in units of $\text{M}^{-1} \cdot \text{cm}^{-1}$. The emission spectra were obtained by exciting the samples, inside a 1 mm optical path cuvette with a 150 W Xenon lamp. The solutions emissions were analyzed with a 0.5 m Spex monochromator and a PMT detector. The signal was amplified by an EG&G 7220 lock-in and processed by a computer.

2.3. Dimerization constants K_D .

HB solutions were prepared in different concentrations from 700 μM up to 5 μM , and their dimerization constants K_D were calculated through Margalit's model¹¹.

2.4. Singlet oxygen generation quantum yields

The infrared emission around 1270 nm, due to the $^1\Delta_g$ transition, was measured using an emission system from Edinburg Instruments. This system is constituted by a Q-switched Nd:YAG laser (continuum Surelite III) emitting 5 ns (FWHM) pulses at 532 nm (second harmonic) at 10 Hz repetition rate, a Hamamatsu R5509 photomultiplier tube and an 1 cm optical path cuvette. A MB solution in ethanol was prepared to be used as reference for the determination of singlet oxygen generation quantum yield ($\Phi_{\Delta}^{\text{MB}} = 0.52$)¹²².

3. Results

3.1 Molar ratios for HB:Ln^{3+} complexes

The Hypocrellin B solution in ethanol exhibits a very large absorption band, extending from 400 nm to 600 nm, which is characterized by three absorption peaks centered at 461, 548 and 589 nm. This observation is in agreement with the fact that the absorption spectra of perylenequinonoid pigments at the visible spectrum consist of three bands, the smaller wavelength and higher intensity being originated in a $\pi \rightarrow \pi^*$ transition, while the others result from intramolecular proton transfer processes¹⁴.

After the addition of lanthanide ions, changes in the HB absorption spectrum occur, indicating that complexation successfully occurred (figure 2).

It can be seen in figure 2 that the lanthanide complexes of HB exhibit absorption peaks similar to those of pure HB, covering the entire visible spectrum. At wavelengths longer than 600 nm, however, the absorptivity is enhanced in a remarkable way. This is good for photodynamic therapy once the longer the incident wavelength, the deeper its penetration in

biological tissues. The best molar ratio for all the analyzed complexes is 1 mol of HB for 2 mols of lanthanide ion, which means that there are two binding sites on HB molecule. The number of bonds of the ions on the molecule is related to the nature of both the HB molecule nature and the metallic ion used.

We propose a model for the structure of the HB:Ln^{3+} complex in which the two metal ions bind to the phenolic hydroxyl oxygens and the carbonyl oxygens of the HB molecule, and the bridge between these ions is done by Cl^- ions.

3.2 Optical characterization of HB:Ln^{3+} complexes

Figure 3 exhibits the samples emission spectra under excitation at 470 nm, and table 1 shows the wavelengths (λ_{max}^E) which correspond to the maximum intensities (I_{470}^{norm}). It can be seen that all solutions show a large emission band, ranging from about 550 to 750 nm, and also that the HB luminescence band seems to be quenched when lanthanide ions are added to the solution.

The shorter wavelength emission band of HB (~620 nm) is due to a $\pi \rightarrow \pi^*$ transition, and the emission band around 690 nm to an intramolecular proton transfer process¹⁴. Regarding the HB molecule complexation by lanthanide ions, modifications in the emission peaks and in the radioactive decay rate constants are observed for all lanthanide used, and the HB shorter wavelength luminescence peak is suppressed with the lanthanide ions addition to the solution. As it is well known, aggregates have its emission peaks shifted to longer wavelengths when compared with its base monomers, and this reinforces the polymeric structure of HB lanthanide complexes=.

3.3. Dimerization constant for HB in ethanol

Figure 4 shows the emission spectra of HB solutions in ethanol for different molar concentrations of HB, and the plotting of $[HB]/L$ as a function of L (Luminescence intensity) for these samples.

The dimerization process can be described by the equation¹¹:



where $[M]$ and $[D]$ are the monomers and dimers concentrations, respectively, and K_D is the equilibrium constant. By expressing the total HB concentration by $[HB]$, and considering that only monomers emit light, the following expression is obtained:

$$\frac{[HB]}{[M]} = 2K_D[M] + 1 \quad (3)$$

$[M]$ can be written as γL , where γ is an experimental coefficient that depends on the wavelength, the specific experimental and recording conditions, and L is the luminescence intensity. Performing this substitution, equation (3) can be rewritten as:

$$\frac{[HB]}{L} = 2K_D\gamma^2 L + \gamma \quad (4)$$

Equation 4 expresses a linear relation between the HB concentration and the luminescence intensity. From emission data obtained under identical experimental and recording conditions, the linear fit of $[HB]$ as a function of L allows the determination of γ and K_D from the axis

crossing point and the line slope. From equation 4 and the fit shown in figure 4b, the dimerization constant K_D obtained for HB is $4.7 \times 10^4 \text{ M}^{-1}$.

3.4. Quantum Yields and Lifetimes of $^1\Delta_g$ state

Figure 5 exhibits the samples emissions in the infrared range (1230 nm up to 1320 nm), under excitation at 532 nm. These spectra were measured with the objective of determining the singlet oxygen generation quantum yields (Φ_Δ) of the samples in ethanol. Methylene blue (MB) was employed as reference ($\Phi_\Delta^{\text{MB}} = 0.52$ ¹²). The emission around 1270 nm is due to the transition from singlet oxygen $^1\Delta_g$ to the ground state (triplet) oxygen $^3\Sigma_g$ ¹⁵:



where h is the Plank's constant and ν is the frequency of the radiation emitted during the transition. Using this relation, $^1\Delta_g$ lifetime (τ_Δ) and singlet oxygen generation quantum yields Φ_Δ were determined from these measurements.

Values of Φ_Δ were calculated using the relation:

$$\Phi_\Delta^S = \frac{I_S}{I_R} \cdot \frac{A_R}{A_S} \cdot \Phi_\Delta^R, \quad (6)$$

where I is the emission intensity at 1270 nm, A is the absorbance in 532 nm and the indexes S and R refer to the sample and reference, respectively.

It may be seen in figure 5a and table 1 that only the lanthanum addition to the HB in ethanol increases the Φ_{Δ} value from 0.47 to 0.62, an enhancement of 32%. Europium addition was responsible for quenching the singlet oxygen generation from 0.47 to 0.03, while terbium addition did not induce remarkable changes in it (from 0.47 to 0.42).

4. CONCLUSIONS

For the first time, to the best of our knowledge, lanthanide complexes of the photosensitizer hypocrellin B were investigated using Optical Spectroscopy. . The lanthanide ions chelate on the HB molecular structure following molar ratio of 2 mols of lanthanide for 1 mol of HB, since these stoichiometry results in the higher molar absorptivity at wavelengths longer than 600 nm, indicating that the HB molecule carries two potential binding sites for lanthanide ions. The dimerization constant for HB was calculated through Margalit's model, and the value $4.7 \times 10^4 \text{ M}^{-1}$ was obtained. The complexes presente emission at longer wavelengths than pure HB, which reveals that the complexes structure is polymeric. Based on these results, a model for the HB complex polymeric structure was proposed. The singlet oxygen generation quantum yields for the complexes were deermined, and the complex of HB with lanthanum ions in ethanol is the only one which enhances the generation of this cytotoxic species.

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Table Captions

Table 1. *Optical properties for HB complexes.* λ_{max}^E is the wavelength which correspond to the maximum emission intensity (I_{470}^{norm}), τ_{Δ} is the $^1\Delta_g$ HB level lifetime and Φ_{Δ} is the singlet oxygen generation quantum yield.

Figure captions

Figure 1. *Molecular structure of hypocrellin B ($C_{30}H_{24}O_9$).*

Figure 2. *Absorption spectra of HB:Lanthanide complexes in ethanol at different molar ratios.*

(a) HB:La, (b) HB:Eu and (c) HB:Tb.

Figure 3. *Emission spectra of HB complexes in ethanol.*

Figure 4. *Emission spectra of (a) HB ethanol solutions for different molar concentrations of HB*

(700 to 5 μ M) and [HB]/L as a function of L for (b) HB.

Figure 5. *Infrared luminescence spectra of samples in ethanol under excitation at 532 nm.*

Table 1

	HB	HB:La³⁺	HB:Eu³⁺	HB:Tb³⁺
$\lambda_{\text{max}}^{\text{E}}$ (nm)	612	650	654	648
I_{470}^{norm} (a.u.)	1.23	0.12	0.02	0.36
τ_{Δ} (μs)	21.88	25.56	32.79	15.61
Φ_{Δ}	0.47	0.62	0.03	0.42

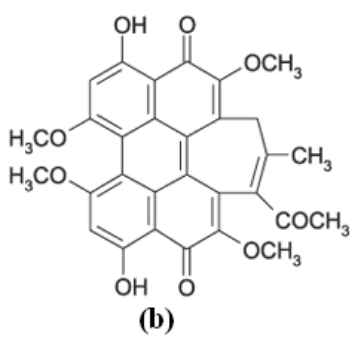


Figure 1

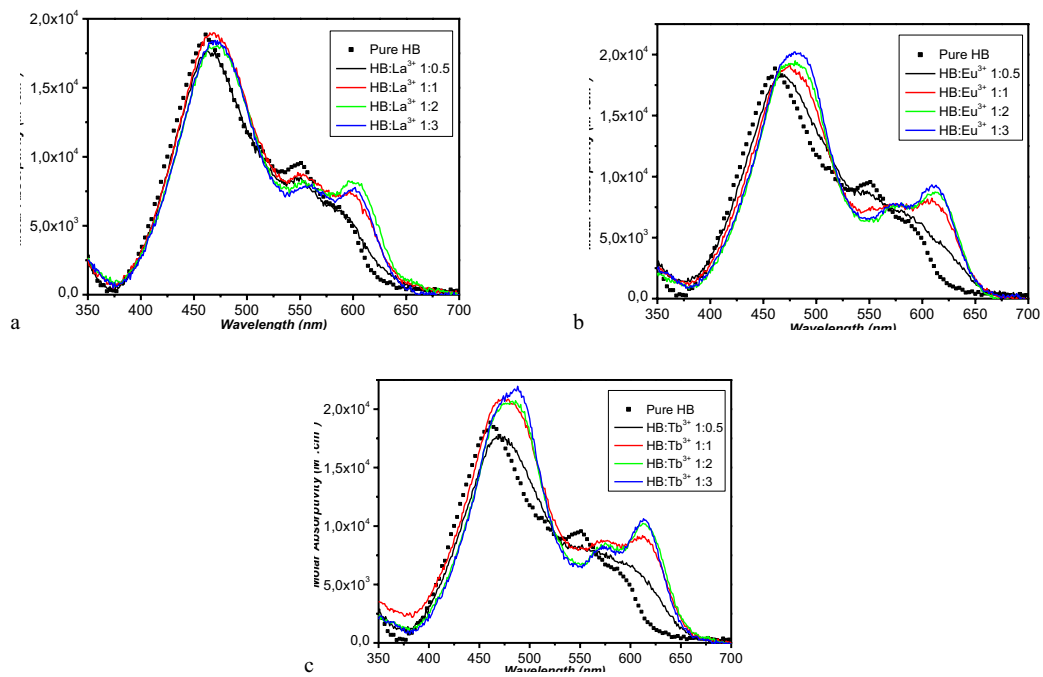


Figure 2

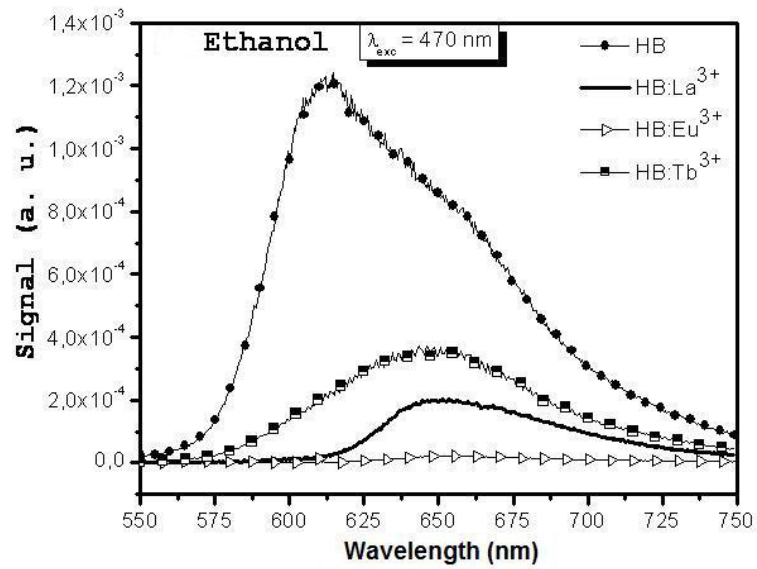


Figure 3.

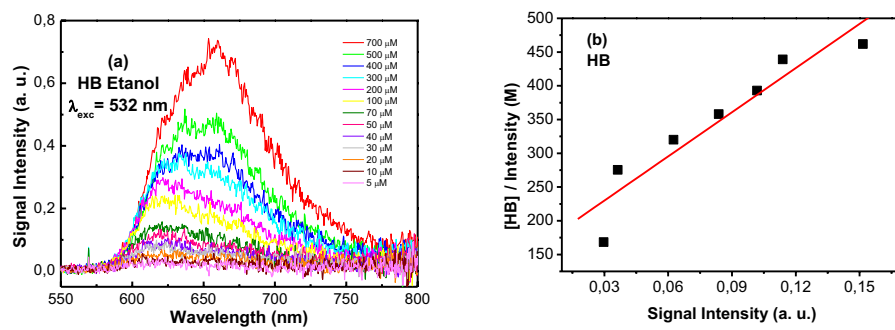


Figure 4.

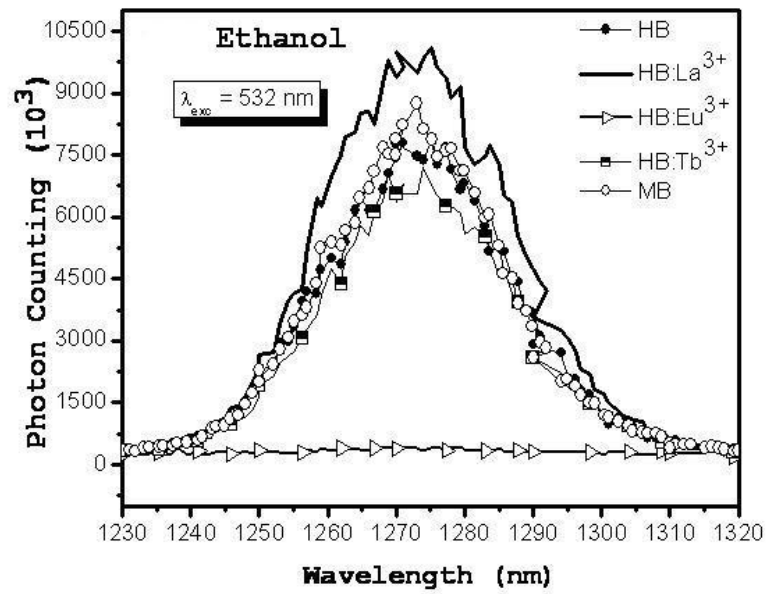


Figure 5.

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